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A quasi-universal medium to break the aerobic/anaerobic bacterial culture dichotomy in clinical microbiology

N. Dione, S. Khelaifia, B. La Scola, J. C. Lagier and D. Raoult

Aix Marseille Université, URMITE, UM63, CNRS 7278, IRD 198, INSERM 1095, Marseille, France

Abstract

In the mid-19th century, the dichotomy between aerobic and anaerobic bacteria was introduced. Nevertheless, the aerobic growth of strictly anaerobic bacterial species such as *Ruminococcus gnavus* and *Fusobacterium necrophorum,* in a culture medium containing antioxidants, was recently demonstrated. We tested aerobically the culture of 623 bacterial strains from 276 bacterial species including 82 strictly anaerobic, 154 facultative anaerobic, 31 aerobic and nine microaerophilic bacterial species as well as ten fungi. The basic culture medium was based on Schaedler agar supplemented with 1 g/L ascorbic acid and 0.1 g/L glutathione (R-medium). We successively optimized this media, adding 0.4 g/L uric acid, using separate autoclaving of the component, or adding haemin 0.1 g/L or α-ketoglutarate 2 g/L. In the basic medium, 237 bacterial species and ten fungal species grew but with no growth of 36 bacterial species, including 22 strict anaerobes. Adding uric acid allowed the growth of 14 further species including eight strict anaerobes, while separate autoclaving allowed the growth of all tested bacterial strains. To extend its potential use for fastidious bacteria, we added haemin for *Haemophilus influenzae, Haemophilus parainfluenzae* and *Eikenella corrodens* and α-ketoglutarate for *Legionella pneumophila.* This medium allowed the growth of all tested strains with the exception of *Mycobacterium tuberculosis* and *Mycobacterium bovis.* Testing primoculture and more fastidious species will constitute the main work to be done, but R-medium coupled with a rapid identification method (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) will facilitate the anaerobic culture in clinical microbiology laboratories.

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Corresponding author: D. Raoult, Aix-Marseille Université, URMITE, UM63 CNRS 7278, IRD 198, INSERM 1095, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille Cedex 5, France
E-mail: didier.raoult@gmail.com

Introduction

Since the discovery of anaerobic bacteria in the mid-19th century by Louis Pasteur, microbiologists have striven to improve techniques for the growth of anaerobic prokaryotes, notably to reduce oxygen tension, without compromising the anaerobic concept [1]. For example, Hungate [2] introduced roll tubes to prepare an anoxygenic medium for methanogenic archaea cultivation. Finegold *et al.* [3] and Moore *et al.* [4] then comprehensively described the anaerobic human flora in the 1970s. This long, fastidious and costly method was gradually abandoned in favour of metagenomic studies [1]. More recently, by culture-dependent studies using dilution method [5] or microbial culturomics [6,7], designing techniques mimicking the natural environment of the bacteria, the human gut repertoire, composed preferentially of anaerobic bacteria, was dramatically extended [8]. Nevertheless, these techniques remain frequently reserved for specialized laboratories.

Otherwise, the ability to grow strictly anaerobic bacterial species in an atmosphere including a low concentration of oxygen was previously reported [9,10]. We recently reported a preliminary study highlighting the aerobic growth of strictly

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anaerobic bacterial species such as *Ruminococcus gnavus* and *Fusobacterium necrophorum* in petri dishes including a basic culture medium supplemented by ascorbic acid and glutathione [11]. Moreover, we demonstrated that the metronidazole susceptibility of strictly anaerobic bacterial species such as *Bacteroides thetaiotaomicron* and *Parvimonas micra* could be tested aerobically [12]. Nevertheless, several other bacterial species, including strictly anaerobic bacteria, were unable to grow in this medium. Here we report the step-by step optimization of this culture medium, aiming to design a quasi-universal culture medium, incubated aerobically, that is largely usable in clinical microbiology laboratories.

Materials and Methods

Bacterial strains tested

Strain types. In this study, we tested 623 bacterial strains from 276 different species, including 82 strictly anaerobic bacterial species, 154 facultative anaerobic bacterial species, 31 aerobic bacterial species, nine microaerophilic bacterial species and ten fungi.

Among them, 542 different strains from 252 species were isolated from ongoing culturomics studies [7], and 81 different strains were from 50 species were isolated in our clinical microbiology laboratory. (Supplementary Table 1(A–E)). All these strains are available in Collection de Souches de l'Unité des Rickettsies (CSUR) collection strains except *Campylobacter coli* and *Campylobacter concisus,* which were available in Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collection strains (Supplementary Table 2). Among the 276 bacterial species tested here, 161 bacterial species corresponded to 99.8% of the positive samples analyzed over 1 year in our clinical microbiology laboratory (Marseille, France). In addition, all the bacteria that we did not test here were isolated less than once per month in our laboratory.

Culture conditions. A growth baseline of all the strains was obtained on the culture condition reference. All the strains were inoculated by spotting in 120 mm petri dishes of R-medium divided into 25 squares. We inoculated each strain from a fresh culture performed in 5% sheep's blood culture incubated under reference culture conditions. The petri dishes were incubated aerobically without CO_2 at 37°C. To determine the conventional growth atmosphere of each strain, we used the Bacterio.Net website (http://www.bacterio.net/), then studied the seminal article describing the bacterial isolate.

Culture medium. For this large study, we used a culture medium that we named R-medium. To design this, we used a basic medium consisting of Schaedler agar (Sigma-Aldrich, Saint-Quentin Fallavier, France) used routinely to cultivate anaerobic bacteria. Thereafter, we added to this medium compounds with an antioxidant activity. The final R-medium 0 consists of Schaedler agar supplemented by 1 g/L ascorbic acid (VWR, Leuven, Belgium) and 0.1 g/L glutathione (Sigma-Aldrich). The pH was adjusted to 7.5 in both culture media basis and antioxidants mix with 10 M KOH before autoclaving. Antioxidants were dissolved in 10 mL distilled water, filtered using 0.2 µm microfilters and added to the autoclaved culture medium stabilized at 50°C. The final culture medium was then poured into 120 mm square petri dishes.

We optimized R-medium 0 by adding 0.4 mg/L uric acid (Sigma-Aldrich), one of the more powerful antioxidants, as previously described [13].

The preparation R-medium 1bis is essentially based on the principle of independently autoclaving the elements that interact during the period of autoclaving to form free radicals, such as the Maillard reaction (between the sugar and the nitrogen source) [14] and the interaction between the phosphate and agar generating H_2O_2, which is an inhibitor of the growth of anaerobic bacteria [15]. To achieve this, three solutions were prepared and autoclaved or filtered independently. Solution A contained 5.67 g of casein hydrolysate proteose, 5 g of peptone and 5 g of yeast extract (Sigma-Aldrich) dissolved in 400 mL of distilled water. The pH was adjusted to 7.5 with 10 M KOH. It was then autoclaved at 121°C for 15 minutes. Solution B consisted of 0.83 g of dipotassium hydrogen phosphate (Merck Santé, Lyon, France), 1.67 g of sodium chloride (Merck Santé), 5.83 g of glucose (MP Biomedicals, Illkirch, France), 0.40 g of L-cysteine (Sigma-Aldrich), 1 g of ascorbic acid (Sigma-Aldrich), 0.4 g of uric acid (Sigma-Aldrich), 0.1 g of glutathione (Sigma-Aldrich), 0.1 g of haemin (Sigma-Aldrich) and 2 g of α-ketoglutarate (Sigma-Aldrich) dissolved in 200 mL of distilled water and filtered using a 0.2 microfilter. Solution C consisted of 15 g of agar (Oxoid, Dardilly, France) dissolved in 300 mL of distilled water. After adjusting the pH to 7.5, the agar solution was autoclaved for 15 minutes at 121°C. The three solutions were stabilized at 56°C after autoclaving and mixed on a hot plate at 56°C. The final culture medium was then poured into 120 mm square petri dishes and stored at 4°C until use.

To increase the culture of fastidious species, we supplemented haemin (0.1 g/L) (Sigma-Aldrich), Schaedler agar containing only 0.01 g/L haemin. The stock solution of haemin was prepared by dissolving 0.1 g haemin (bovine haemin chloride) (Sigma-Aldrich) in 1 mL of NaOH. This allows better solubilization, as previously described [16].

We added α-ketoglutarate (2 g/L) (Sigma-Aldrich) to allow the growth of *Legionella pneumophila.* Indeed, α-ketoglutarate is

a main component included in buffered charcoal yeast extract, the reference medium for *L. pneumophila* growth [17].

Culture baseline, and positive and negative controls. As positive control, and to obtain a baseline growth before inoculation on R-medium, we used 5% sheep's blood agar (bioMérieux, Marcy l'Étoile, France) for all the strains except for *Haemophilus* spp. (chocolate agar; BD, Pont de Claix, France) and for *L. pneumophila* (buffered charcoal yeast extract agar; BD). The strains were incubated in their respective atmosphere condition (aerobe, anaerobe, microaerophile conditions) at 37°C without CO_2. We used AnaeroGen (Thermo Scientific, Dardilly, France) transparent incubation jars with generators and Campygen (Oxoid, Dardilly, France) for anaerobes and microaerophiles bacteria, respectively.

Negative controls were incubated onto Schaedler agar medium (Sigma-Aldrich) without antioxidants and incubated aerobically without CO_2 at 37°C. Noninoculated agar was also incubated under aerobic and anaerobic conditions and introduced as a sterility control. All experiments reported here were performed in triplicate.

Growth detection and identification

We checked for growth daily *via* detection by the naked eye. All the colonies were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) (Bruker Daltonics, Leipzig, Germany). We used MALDI Biotyper 3.0 software to compare the spectra to our homemade MALDI-TOF database, as previously reported [18].

Results

We were able to grow aerobic, anaerobic and microaerophilic nonfastidious bacterial species on the same culture medium in aerobic atmosphere (excluding fastidious bacteria).

In R-medium 0, containing ascorbic acid and glutathione, we successfully aerobically cultured 237 bacterial species including 60 strictly anaerobic bacterial species, 146 facultative anaerobes and 31 aerobes (Supplementary Table 1(A–C)). We failed to grow 42 strains from 30 different bacterial species (10.8%) including 22 strictly anaerobic bacterial species, two facultative anaerobes and six microaerophilic bacteria (Table 1).

We optimized the medium by the addition of uric acid (i.e. R-medium 1) and tested 51 strains from 36 different bacterial species, including all the previous failures. This media allowed the aerobic growth of 20 supplementary strains from 14 bacterial species including eight strict anaerobes (four *Clostridium* spp., *Blautia coccoides, Peptoniphilus asaccharolyticus, Odoribacter splanchinus* and *Tissiriella preacuta*), one facultative anaerobe (*Gardnerella vaginalis*) and five microaerophilic *Campylobacter* spp. (Table 1). Nevertheless, 14 strict anaerobes (*Acidaminococcus intestini,* four *Alistipes* species, two *Anaerosalibacter* species, three *Collinsella* species, *Dielma fastidiosa, Holdemania massiliensis, Parvimonas micra* and *Prevotella buccalis*), one facultative anaerobe (*Corynebacterium suicordis*) and one microaerophile (*Vagococcus fluvialis*) did not grow in this culture media (Table 1, Fig. 1).

Finally, in order to increase the culture of anaerobic bacteria, we used the medium containing the three antioxidants

TABLE 1. List of failed and successful growth of bacterial strains including strict anaerobes, facultative anaerobes and microaerophiles but excluding fastidious bacterial species[a]

Characteristic	Strict anaerobes		Facultative anaerobes	Microaerophiles
Bacteria that did not grow in R-medium 0 (glutathione + ascorbic acid)	*Acidaminococcus intestini*	*Clostridium subterminale*	*Corynebacterium suicordis*	*Campylobacter coli*
	Alistipes finegoldii	*Collinsella aerofaciens*	*Gardnerella vaginalis*	*Campylobacter concisus*
	Alistipes indistinctus	*Collinsella massilioamazoniensis*		*Campylobacter cuniculorum*
	Alistipes putredinis	*Collinsella tanakaei*		*Campylobacter fetus*
	Alistipes shahii	*Dielma fastidiosa*		*Campylobacter jejuni*
	Anaerosalibacter bizertensis	*Holdemania massiliensis*		*Vagococcus fluvialis*
	Anaerosalibacter massiliensis	*Odoribacter splanchnicus*		
	Blautia coccoides	*Parvimonas micra*		
	Clostridium baratii	*Peptoniphilus asaccharolyticus*		
	Clostridium lituseburense	*Prevotella buccalis*		
	Clostridium ramosum	*Tissierella praeacuta*		
Bacteria that did not grow in R-medium 1 (glutathione + ascorbic acid + uric acid)	*Acidaminococcus intestini*	*Collinsella aerofaciens*	*Corynebacterium suicordis*	*Vagococcus fluvialis*
	Alistipes finegoldii	*Collinsella massilioamazoniensis*		
	Alistipes indistinctus	*Collinsella tanakaei*		
	Alistipes putredinis	*Dielma fastidiosa*		
	Alistipes shahii	*Holdemania massiliensis*		
	Anaerosalibacter bizertensis	*Parvimonas micra*		
	Anaerosalibacter massiliensis	*Prevotella buccalis*		

[a]All listed bacterial species grew in R-medium 1bis (glutathione + ascorbic acid + uric acid) made by separate autoclavage; 584 bacterial strains from 237 bacterial species grew in R-medium 0.

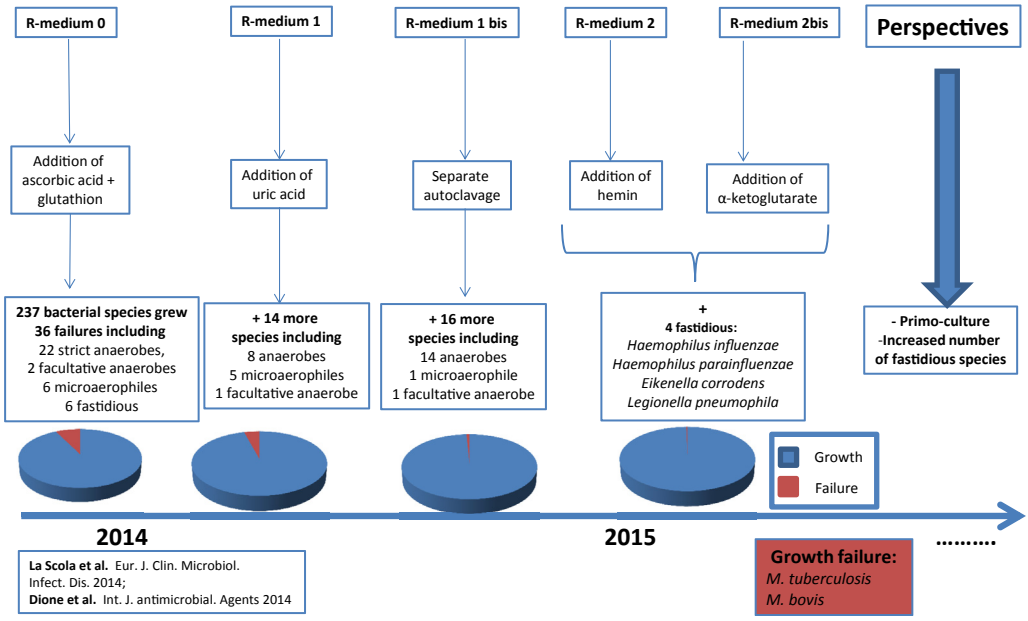

FIG. 1. Flowchart of optimization of R-medium.

prepared with separate autoclaving (i.e. R-medium 1bis). All the 16 bacterial species that did not grow in R-medium 1 grew in this medium, including 14 strictly anaerobic bacterial species, the facultative anaerobe species and the microaerophilic bacterial species (Table 1, Fig. 1).

Growing fastidious bacteria and fungi

In order to propose a quasi-universal culture medium, we tested some fastidious bacterial species in R-medium (Supplementary Table 1(D, E)). Among these, *Francisella tularensis* required uric acid, growing in R-medium 1. *Bartonella henselae* and *Mycobacterium smegmatis* required separate autoclaving, growing in R-medium 1bis.

Six other species (*Haemophilus influenzae, Haemophilus parainfluenzae, Eikenella corrodens, Mycobacterium tuberculosis, Mycobacterium bovis* and *L. pneumophila*) were tested in a supplemented medium. In the medium supplemented with haemin (R-medium 2bis), *H. influenzae, H. parainfluenzae* and *E. corrodens* grew. In the medium supplemented with haemin and α-ketoglutarate, we observed the growth of *L. pneumophila* but not the growth of *M. bovis* and *M. tuberculosis.*

We also grew ten yeast species in R-medium 0. *Candida albicans, Candida glabrata, Candida krusei, Candida kefyr, Candida lusitaniae, Candida tropicalis, Candida parapsilosis, Candida orthopsilosis, Candida dubliniensis* and *Saccharomyces cerevisiae* all grew after 24 hours of incubation (Supplementary Table 1(E)).

Overall results

Among the 633 bacterial and fungal strains tested aerobically in R-medium, we failed to achieve the growth of only *M. tuberculosis* and *M. bovis.* Among them, all the 135 strictly anaerobic bacterial strains, belonging to four phyla, 30 different genera and 82 different species, were successfully cultured. These included 28 *Clostridium* species (including *C. difficile* and *C. perfringens*), ten *Bacteroides* species (including *B. thetaiotaomicron, B. vulgatus, B. uniformis* and *Bacteroides fragilis*), four *Alistipes* species (including *A. finegoldi* and *A. shahii*), three *Collinsella* species, three *Peptoniphilus* species, two *Fusobacterium* species (*F. necrophorum* and *F. nucleatum*), two *Veillonella* species, *Parvimonas micra, Prevotella buccalis* and *Finegoldia magna.* All grew within 48 to 96 hours of incubation (Supplementary Table 1(A)). Using Schaedler agar without antioxidants incubated aerobically as negative control, there was no growth of the strictly anaerobic, microaerophilic and fastidious bacterial strains incubated aerobically at 37°C without CO_2.

Discussion

To our knowledge, we report here the largest study of the aerobic growth of bacterial and fungal species of clinical interest, in a versatile culture medium with occasional slight necessary modification to the medium (Fig. 2). Our results are robust because we used a large number of bacterial strains isolated from humans [8], available from a collection of bacterial strains (CSUR or DSMZ; Supplementary Table 2). Furthermore, we used both negative and positive controls; all the strains were identified by MALDI-TOF in order to exclude contamination [18]. We voluntarily chose the simplest incubation atmosphere (aerobic condition without CO_2). Finally,

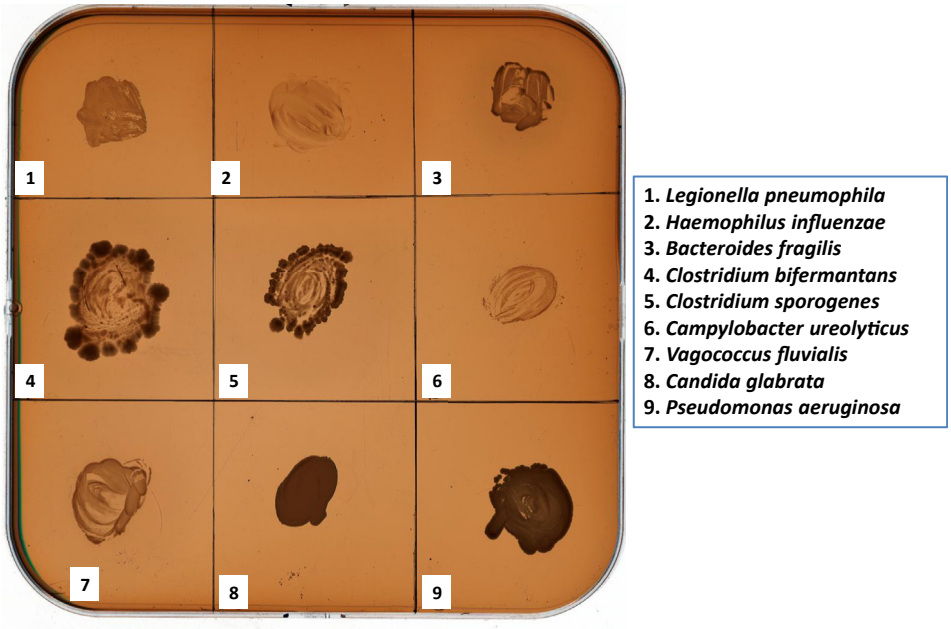

FIG. 2. Impossible petri dish. Concomitant subculture was achieved in same culture medium (R-medium 2bis) of three strict anaerobic species (*Bacteroides fragilis, Clostridium bifermentans, Clostridium sporogenes*), two fastidious species (*Legionella pneumophila, Haemophilus influenzae*), two microaerophiles species (*Vagococcus fluvialis, Campylobacter ureolyticus*), one strict aerobe species (*Pseudomonas aeruginosa*) and one fungal species (*Candida glabrata*).

the aerobic culture of strictly anaerobic bacterial species is not surprising when we consider the fact that bacteria considered to be strict anaerobes grew easily in human blood during bacteraemia despite being composed of a large amount of oxygen [19].

The addition of some components dramatically extended the spectra of the growing anaerobic bacteria, as previously described [11,12]. As an example, uric acid, although it has antioxidant properties under certain conditions, is not conventionally used in microbiology yet enabled the growth of a further eight anaerobic bacterial species [13]. As previously demonstrated by Tanaka *et al.* [15] with *Gemmatimonas aurantiaca,* one of the hidden pitfalls that could support a part of the "great plate count anomaly" is explained by the larger number of colonies observed in petri dishes when certain compounds were autoclaved separately and then mixed before solidification of the media, in comparison with when all components were autoclaved together. We used this separate autoclaving process to make our medium including the three antioxidants, to enable the growth of 14 strict anaerobic bacterial species which initially resisted growing in R-medium 1. Finally, to increase the growth of fastidious bacteria, with the supplementation of the medium with haemin, a component commonly used in culture media [17], some fastidious bacteria grew and the addition of α-ketoglutarate, a component included in buffered charcoal yeast extract, the reference medium for *L. pneumophila,* allowed its growth [17].

This culture medium, coupled with rapid identification by MALDI-TOF, holds great potential in clinical microbiology, greatly simplifying procedures and reducing costs [1,20,21]. In the future, automatic incubation chambers of such media coupled with automatic colony picking for identification by MALDI-TOF will deepen this research field. Our study could be strengthened by showing R-medium can support culture of primary anaerobic isolates, which will constitute the next step to be performed through large-scale culture studies directly from clinical specimens. Further reducing the incubation time for some strict anaerobes will be among the future challenges to overcome. When confirmed, this new strategy may make it possible to do away with costly equipment (anaerobic chambers, anaerobic jars, roll-tube, etc.) and make it possible for any laboratory to grow "anaerobic" bacteria. This significant development occurs in parallel with a dramatic rebirth of culture in both environmental microbiology [22] and clinical microbiology, as recently reported notably in a gut microbiota study [1,7,23]. This was highlighted by both the increased number of officially acknowledged bacterial species and the increased number of recognized pathogenic species in humans, as reported in a review that counted 2172 bacterial species cultured at least once in humans [8].

In conclusion, our study illustrates the blindness from which we sometimes suffer and the difficulties to move beyond notions that we consider indisputable, such as the aero/anaerobic dichotomy.

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Transparency Declaration

SK, BL and DR are coinventors of a patent on the culture of anaerobic bacteria (1H53 316 CAS 9 FR). The other authors report no conflicts of interest relevant to this article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.10.032.

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